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Daily acute intermittent hypoxia elicits functional recovery of diaphragm and inspiratory intercostal muscle activity after acute cervical spinal injury

A. Navarrete-Opazo^{1,2}, S Vinit^{1,3}, B.J. Dougherty¹, and G.S. Mitchell^{1,4}

¹Department of Comparative Biosciences University of Wisconsin-Madison, Madison, WI, USA, 53706

Abstract

A major cause of mortality after spinal cord injury is respiratory failure. In normal rats, acute intermittent hypoxia (AIH) induces respiratory motor plasticity, expressed as diaphragm (Dia) and second external intercostal (T2 EIC) long-term facilitation (LTF). Dia (not T2 EIC) LTF is enhanced by systemic adenosine 2A (A_{2A}) receptor inhibition in normal rats. We investigated the respective contributions of Dia and T2 EIC to daily AIH-induced functional recovery of breathing capacity with/without A_{2A} receptor antagonist (KW6002, i.p.) following C₂ hemisection (C₂HS). Rats received daily AIH (dAIH: 10, 5-min episodes, 10.5% O₂; 5-min normoxic intervals; 7 successive days beginning 7 days post-C₂HS) or daily normoxia (dNx) with/without KW6002, followed by weekly (reminder) presentations for 8 weeks. Ventilation and EMGs from bilateral diaphragm and T2 EIC muscles were measured with room air breathing (21% O₂) and maximum chemoreceptor stimulation (MCS: 7% CO₂, 10.5% O₂). dAIH increased tidal volume (V_T) in C₂HS rats breathing room air (dAIH + vehicle: 0.47 ± 0.02, dNx + vehicle: 0.40 ± 0.01ml/100 g; p<0.05) and MCS (dAIH + vehicle: 0.83 ± 0.01, dNx + vehicle: 0.73 ± 0.01ml/100g; p<0.001); KW6002 had no significant effect. dAIH enhanced contralateral (uninjured) diaphragm EMG activity, an effect attenuated by KW6002, during room air breathing and MCS (p<0.05). Although dAIH enhanced contralateral T₂ EIC EMG activity during room air breathing, KW6002 had no effect. dAIH had no statistically significant effects on diaphragm or T2 EIC EMG activity ipsilateral to injury. Thus, two weeks post-C₂HS: 1) dAIH enhances breathing capacity by effects on contralateral diaphragm and T2 EIC activity; and 2) dAIH-induced recovery is A_{2A} dependent in diaphragm, but not T2 EIC. Daily AIH may be a useful in promoting functional recovery of breathing capacity after cervical spinal injury, but A_{2A} receptor antagonists (eg. caffeine) may undermine its effectiveness shortly after injury.

Corresponding Author: Gordon S. Mitchell, Department of Physical Therapy, College of Public Health & Health Professions, University of Florida, 1225 Center Drive, PO Box 100154, Gainesville, FL, 32610, Phone: 352-273-6107, Department Fax: 352-273-6119, gsmitch@phhp.ufl.edu.

²Current address: Teletón Children Rehabilitation Institute, Alameda 4620, Santiago, Chile

³Current address: Université de Versailles Saint-Quentin-en-Yvelines, Unité Endicap, UFR des Sciences de la Santé - Simone Veil, Montigny-le-Bretonneux, France.

⁴Current address: Department of Physical Therapy, University of Florida, Gainesville, FL, USA 32610

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Keywords

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Introduction

A major life-threatening consequence of cervical spinal cord injury (SCI) is the disruption of brainstem-spinal cord projections that give rise to spinal respiratory motor neuron activity and breathing (Winslow and Rozovsky, 2003). One approach to enhance respiratory function in SCI patients is to induce plasticity in spared synaptic pathways to respiratory motor neurons (Dale et al., 2014; Mitchell, 2007; Ramer et al., 2000). Moderate acute intermittent hypoxia (AIH) induces respiratory motor plasticity (Devinney et al., 2013; Feldman et al., 2003; Mahamed and Mitchell, 2007; Mitchell et al., 2001), strengthening synaptic inputs to phrenic motor neurons (Fuller et al., 2003; Golder and Mitchell, 2005; Lovett-Barr et al., 2012). The functional consequences of this plasticity are: 1) persistent increases in phrenic nerve activity in anesthetized rats (phrenic long-term facilitation, pLTF) (Bach and Mitchell, 1996); 2) diaphragm (diaLTF) and second thoracic external intercostal (T2 EIC LTF) muscle long-term facilitation in unanesthetized rats (Navarrete-Opazo and Mitchell, 2014); and 3) ventilatory LTF (largely tidal volume; vLTF) in unanesthetized rats (Nakamura et al., 2010; Olson et al., 2001) and humans (Pierchala et al., 2008; Tester et al., 2014). Thus, repetitive AIH may be a viable strategy to restore function in respiratory muscle activity and breathing capacity (Dale et al., 2014).

Multiple, distinct cellular mechanisms elicit AIH-induced respiratory motor plasticity, including serotonin- and adenosine-dependent pathways (Dale-Nagle et al., 2010; Devinney et al., 2013; Nichols et al., 2012). These serotonin and adenosine-dependent pathways interact via cross-talk inhibition (Dale-Nagle et al., 2010; Hoffman et al., 2010). With moderate AIH, pLTF is serotonin-dependent, and is constrained by concurrent, sub-threshold activation of spinal A_{2A} receptors; thus, spinal and systemic A_{2A} receptor inhibition enhance pLTF in anesthetized rats (Hoffman et al., 2010) and diaLTF in unanesthetized rats (Navarrete-Opazo et al., 2014).

Although it may seem logical to hypothesize that A_{2A} receptor inhibition would enhance the therapeutic benefits of repetitive AIH, different (serotonin- and adenosine-dependent) cellular mechanisms may underlie repetitive AIH-induced functional recovery in rats with acute *versus* chronic spinal injuries. For example, shortly after C₂ spinal hemisections (C₂HS), serotonergic innervation of the phrenic motor nucleus is diminished (Saruhashi et al., 1996; Zhou and Goshgarian, 1999), abolishing serotonin-dependent pLTF two weeks post-C₂HS (Golder and Mitchell, 2005). However, with time post-injury, serotonergic innervation and the capacity for serotonin-dependent pLTF recover (>8 weeks post-C₂HS). Similarly, neither AIH-induced diaphragm nor T2 EIC LTF are expressed 7 days post-C₂HS in unanesthetized rats, yet both can be elicited 8 weeks post-injury (Navarrete-Opazo et al., 2014). Although serotonin-dependent respiratory plasticity is compromised two weeks post-

C₂HS, the capacity for A_{2A} receptor induced phrenic motor facilitation remains (Golder et al., 2008).

Moderate daily AIH (dAIH) partially restores breathing capacity 2 weeks post-C₂HS (Lovett-Barr et al., 2012). With this acute SCI, functional recovery may nevertheless have arisen from serotonin-dependent mechanisms since more hypoxic episodes were used in this study (10 episodes/day for 7 days versus 3 episodes in a single day) (Golder and Mitchell, 2005; Lovett-Barr et al., 2012). An alternative hypothesis is that more hypoxic episodes and/or more severe hypoxemia within episodes triggered distinct, A_{2A} receptor-dependent recovery of respiratory function (Nichols et al., 2012). These alternatives can be discriminated by the response to systemic A_{2A} receptor antagonist pre-treatment. In the former case, we predict enhanced dAIH-induced functional recovery with A_{2A} receptor inhibition; in the latter case, the opposite result will be observed (attenuated dAIH-induced functional recovery). The respiratory effects of combined A_{2A} receptor inhibition and dAIH 2 weeks post-C₂HS have not been explored. Our working hypothesis is that dAIH elicits plasticity and functional recovery via adenosine-dependent mechanisms with acute SCI, but reverts to serotonin-dependent (adenosine-constrained) mechanisms with chronic injury. Thus, we predicted that A_{2A} receptor antagonist pretreatment impairs dAIH-induced recovery of breathing capacity in acute (2 week) C₂HS rats.

Repetitive AIH also improves non-respiratory motor functions after SCI (Hayes et al., 2013; Lovett-Barr et al., 2012; Trumbower et al., 2012), and this functional improvement is greatest when AIH is paired with task-specific training (Hayes et al., 2013). The grooming test (Bertelli and Mira, 1993) utilizes stereotypical, brainstem regulated grooming to evaluate somatic motor recovery following injury. This innate behavior (grooming) does not require pre-training and results are unaffected by repeated testing (Berntson et al., 1988; Bertelli and Mira, 1993).

The duration of functional improvement following repetitive AIH has not been determined. Daily AIH-induced respiratory functional recovery lasts at least 1 day following dAIH (Lovett-Barr et al., 2012), and preliminary data suggest that effects last at least one week post-dAIH (Terada, Vinit, MacFarlane and Mitchell, unpublished). We wondered if weekly AIH “reminders” extend dAIH-induced functional recovery. Such reminders have been effective at prolonging behavioral memories (Martin et al., 2010; Rovee-Collier et al., 1980; Wiltgen and Tanaka, 2013).

Here we studied the impact of dAIH and systemic A_{2A} receptor inhibition on respiratory and non-respiratory motor function in unanesthetized rats following acute C₂HS. We measured ventilation with whole-body plethysmography, bilateral diaphragm and T2 external intercostal muscle activity via electromyography (EMG) radiotelemetry, and grooming behavior to evaluate a non-respiratory motor function. We tested the hypotheses that, two weeks post-C₂HS: 1) dAIH improves breathing capacity (i.e. tidal volume) and grooming behavior; 2) spontaneous T2 EIC motor recovery ipsilateral to injury is greater than in diaphragm; 3) dAIH enhances the ability to increase diaphragm and T2 EIC muscle activity during maximal chemoreceptor stimulation; 4) systemic A_{2A} receptor antagonist administration before daily AIH impairs functional recovery of breathing capacity and

diaphragm muscle activity; and 5) weekly AIH presentations (single day) prolong dAIH-induced functional benefits.

Methods

Animals

All experiments began with 3-4 months old, male Sprague-Dawley rats (310-440 g, colony 211, Harlan, Indianapolis, IN). Rats were individually housed in a controlled environment (12-h light/dark cycle). The Animal Care and Use Committee at the School of Veterinary Medicine, University of Wisconsin approved all experimental procedures in this study.

Experimental preparation

Surgical preparation—For telemetry implantation and C₂ cervical hemisection, sterile surgery was performed under isoflurane anesthesia (in 100% O₂). The rats were injected with buprenorphine (0.03 mg/kg), carprofen (Rimadyl, 5 mg/kg) and enrofloxacin (Baytril, 4 mg/kg) subcutaneously to minimize potential post-operative pain and infection. Body temperature was maintained at 36.5-37.5°C using a rectal probe and external heating pad. A cannula was inserted into the trachea and the rats were artificially ventilated (tidal volume, 2.0-2.5 ml; Rodent Ventilator, model 683; Harvard Apparatus, South Natick, MA) with 1.5-2.5% isoflurane in 100% O₂ during surgery. Effective anesthesia was confirmed by abolition of pedal withdrawal and corneal blink reflexes. Oxygen saturation was monitored by pulse oximetry (model 8600; Nonin Medical Inc. Plymouth, MN) during surgery. At the end of surgery buprenorphine, carprofen and enrofloxacin (see above for dosage) were administered at 12 h intervals for 48 h post-surgery. Rats were visually monitored and weighed daily. In spinally injured rats, post-surgical care included trimming nails after surgery, and cleaning fur, eyes and snout with warm water daily for 7 days to avoid accumulation of porphyrin. Rats had free access to pellets and high caloric nutritional gels inside their cages. In telemetry implantation and spinal injury surgeries, stainless steel staples were used to close the wound, and were removed 7 days post-surgery.

Telemetry transmitter implantation—After anesthetic induction, rats were placed in a supine position and the ventral surface of the abdominal muscle exposed. A sterilized transmitter (model 4ET-S1/2; Data Sciences International [DSI], St. Paul, MN) was inserted into the peritoneal cavity. The transmitter allowed simultaneous and continuous monitoring of electrical bio-potentials, body temperature and general locomotor activity. In the present study, the four bio-potential channels were used to record electromyographic (EMG) activity from bilateral diaphragm and second external intercostal (T2 EIC) muscles. Implantations were performed as follows. First, both right and left hemi-diaphragms were exposed through a midline incision. On both hemidiaphragms, two leads were implanted on the mid-costal area using a 23-G syringe needle guide and tissue adhesive (Vetbond 1469SB; 3M Animal care product, St. Paul, MN) as reported in previous studies (Terada and Mitchell, 2011). Next, right and left T2 EIC muscles were exposed through a 2.5 cm mid-sternum incision, starting in the upper edge of sternum, followed by retraction of pectoralis major and minor on the right and left side. The right and left T2 EIC muscles were implanted 1.0 cm right and left from the sternum respectively and the second interspace was identified by counting from

the first interspace. The bi-potential lead pairs targeting T2 EIC muscles were tunneled subcutaneously from the body of the transmitter placed in the peritoneal cavity. As used in the diaphragm, all T2 EIC leads were implanted using a 23-G syringe needle guide and tissue adhesive to keep the leads on place. Finally, abdominal muscles and pectoralis major were sutured in the midline with polysorb 3.0 and the skin was closed with wound staples in both ventral thorax and abdomen.

Cervical C₂ hemisection—One week after telemetry implantation, spinal hemisections at the second cervical level (C₂HS) were performed. The surgical technique was consistent with previous reports (Dougherty et al., 2012b; Fuller et al., 2009; Vinit et al., 2009). After appropriate anesthesia and pre-operative care, the spinal cord was exposed at C₂ via a dorsal laminectomy. The dura matter was cut and a left C₂ hemisection (C₂HS) performed using a micro-scalpel followed by aspiration. The overlying muscles were sutured with polysorb 3.0 and the skin closed with stainless steel wound clips. Sham rats underwent cervical laminectomy without spinal injury.

Whole-body plethysmography—Rats were placed individually in a 4 L DSI Plethysmography chamber (model 600-1211-001). Pressurized air flowed through the chamber at 4L/min, allowing control of inspired gas composition. The chamber was positioned onto a receiver (see below for telemetry signal acquisition) to measure EMG and plethysmography simultaneously. Compensated whole body plethysmography was used to assess tidal volume (V_T); compensation was based on chamber temperature (model P/N 60-1210-001) and humidity sensors (model P/N 600-1211-001). The system used a transducer (Buxco, model TRD5700) and a gas analyzer (CWE, Gemini). Plethysmography data were analyzed in 1 min bins during baseline (i.e. normoxia, 20 minutes) and during maximum chemoreceptor stimulation (hypoxia: 10.5% O₂ and hypercapnia: 7% CO₂; 20 min). To obtain more accurate V_T assessments, we used intraperitoneal temperature from the telemetry system to compensate for body temperature changes.

Telemetry signal acquisition—For the AIH protocol (see below), rats were placed in custom-made Plexiglas chambers positioned on receivers (model RPC-2; DSI, St. Paul, MN). Signals from the implanted radiotelemetry transmitter were detected by the receivers and sent to a data exchange matrix (model ACQ-7700; DSI, St Paul, MN). Four channels of EMG, body temperature and general locomotor activity in unanesthetized freely moving rats were monitored during experiments on a laboratory computer (data acquisition system: PONEMAH Physiology Platform; DSI, St. Paul, MN). EMG signals were sampled at 1200 Hz and analyzed with Neuroscore software (DSI, St. Paul, MN).

Grooming Test—Forelimb grooming function was assessed using a scoring system adapted from Bertelli and Mira (Bertelli and Mira, 1993), originally developed to examine recovery in a rat brachial plexus reconstruction model. Cool tap water was applied to the rat's head and back with soft gauze, and the rat was returned to the chamber. Grooming activity was recorded with a video camera from the onset of grooming through at least two stereotypical grooming sequences (~2 min), which include: 1) licking of the forepaws and face washing, 2) forelimb grooming of the face, 3) repetitive licking of the body, and 4)

hindpaw scratching (Berntson et al., 1988). Scoring was done as illustrated in Fig.8. Slow motion video playback was used to score each forelimb independently by the maximal contact made while initiating any part of the grooming sequence. The animals were tested after telemetry implantation, after spinal cord injuries, and weekly after dAIH exposure until the end of the study.

Drug preparation—KW6002 (Istradefyline, Sigma-Aldrich) is a selective adenosine A_{2A} receptor antagonist with a molecular weight of 384 and a K_i of 29.6 nM in rats. It's half-life of 110 min, 97% bio-availability after intraperitoneal injection, and ability to cross the blood brain barrier (Yang et al., 2007), make it a suitable drug for our *in vivo* experiments. The drug was dissolved in DMSO at 9.3 mg/ml, sonicated and stored at 4°C in a dark vial protected from light. The day of the experiment, the drug was administered via intraperitoneal injection at a dose of 0.5 mg/kg.

Experimental design—Five days after telemetry implantation, grooming tests were performed, and then simultaneous plethysmography and EMG recordings were made during baseline air breathing (Nx) and maximum chemoreceptor stimulation (MCS) to establish pre-C₂HS values. One day after C₂HS, the same protocols were done to demonstrate a complete hemisection.

Seven days post-C₂HS, 32 rats were randomly allocated into the following groups: 1) dAIH + KW6002 (n=8), 2) dAIH + vehicle (n=8); 3) dNx + KW6002 (n=6); 4) dNx + vehicle (n=6); and 5) Sham (n=4). Group 1 rats received an intraperitoneal injection of KW6002 (0.5 mg/kg) 5 minutes before AIH on 7 consecutive days starting day 7 post-C₂HS, and then weekly presentations of AIH with KW6002 (3-7 weeks post-C₂HS). Group 2 rats received the same protocol, but KW6002 was replaced with vehicle (DMSO). During the same time window, rats in groups 3 and 4 (time control rats) were exposed to normoxia and KW6002 or vehicle, respectively. Sham rats were exposed to normoxia and did not receive intraperitoneal injections. All groups were compared over time through assessment of weekly video recording of grooming test performance, and simultaneous plethysmography and EMG recordings during normoxia and MCS until the end of the study (see fig.1, experimental design).

Acute intermittent hypoxia—Normoxic (21% O₂) and hypoxic (10.5% O₂) conditions were established in custom-made chambers (Plexiglas cylinder, 12 × 4 inches id; 1 rat per chamber) by mixing O₂ and N₂ gas with a custom-made computer-controlled mass-flow controller system to obtain the desired inspired oxygen concentrations. Within the chambers, CO₂ and O₂ levels were continuously monitored during the entire protocol (O₂ Analyzer, model 17518; CO₂ Analyzer, model 17515; VacuMed Inc, Ventura, CA). Gas flowed through the chamber at a rate of 4 L/min, keeping chamber CO₂ concentration less than 0.5% at all times. 95% of the change in O₂ levels within hypoxic episodes was achieved in 25 ± 5 sec. At 8:00 am, on the experimental day, rats were placed in the chamber for 2-hour acclimation. Next, intraperitoneal injections of either KW6002 or vehicle were administered accordingly (see experimental design). Once all rats were in the chambers, the experimental groups were administered the AIH protocol (10, 5-min 10.5% O₂ interspersed with 5-min 21% O₂, for a total of 95 min). Control and sham rats were administered

continuous normoxia (i.e. time controls; TC). Chamber temperature was kept at 22.5-24.5°C during the entire protocol.

Maximum chemoreceptor stimulation—Normoxic (21% O₂), hypoxic (10.5% O₂) and hypercapnic conditions (7% CO₂) were established in plethysmography chambers (see above) by mixing O₂, N₂ and CO₂ gas via a custom-made, computer-controlled mass flow controller system to obtain the desired inspired gas concentrations. After 30 min acclimation, baseline breathing during normoxia was recorded for 20 min, followed by 20 min of maximum chemoreceptor stimulation (MCS) (10.5% O₂ and 7% CO₂, 20 min). This protocol enables assessment of a standardized, high breathing level elicited by maximal chemoreflex activation (Navarrete-Opazo and Mitchell, 2014). EMGs and whole-body plethysmography were used to measure tidal volume (V_T) and breathing frequency, along with respiratory muscle EMG signals.

Tissue Processing—To verify the extent of hemisections, each spinal cord was removed after completion of the experiments, immersed in paraformaldehyde (4%, overnight at 4°C) and cryoprotected in increasing concentrations of sucrose (20–30%). Tissues were then frozen in isopentane (−45°C) and stored at −80°C. Longitudinal sections of the spinal cord (C1 to C6, 30 μm thick) were stained with cresyl violet and examined histologically with light microscopy to reconstruct the injury in a transverse plane (Vinit et al., 2006), according to Paxinos and Watson (Paxinos and Watson, 1998). We used ImageJ software (National Institute of Health; <http://rsb.info.nih.gov/ij/>) to measure and compare the extent of the hemisection among groups.

Data analyses

EMG signals were analyzed with Neuroscore software. The raw signal was filtered (100-624 Hz), rectified, integrated (100 msec) and averaged for each muscle. EMG values during active locomotor activity and grooming behavior were excluded in the analysis. Absolute values of tidal volume and breathing frequency were averaged. EMG amplitudes in each muscle were expressed as a percent change from pre-injury values. Grooming test score (0-5) was assessed separately in right and left forelimb.

All variables were compared among groups, and statistical inferences were made using two-way, repeated measures ANOVA for time (baseline and MCS) and treatment (see experimental design); individual comparisons were made using Fisher's LSD *post hoc* tests (Sigma-Stat version 2.03, Systat Software Inc, San Jose, CA, USA). Differences indicated as statistically significant were $P < 0.05$. All values are expressed as means \pm SEM.

Results

Daily acute intermittent hypoxia enhances capacity to increase tidal volume

Pre-injury values were established 5 days post-telemeter implantation (Fig.1) in uninjured rats exposed to 20 min of normoxia followed by 20 min of MCS for both plethysmography and EMG recordings. One day post-C₂HS, all experimental groups exhibit significant reductions in tidal volume versus pre-injury and sham values when breathing air (Sham:

0.55 ± 0.02 vs. dAIH + vehicle: 0.38 ± 0.01, dAIH + KW6002: 0.39 ± 0.02, dNx + vehicle: 0.40 ± 0.02, dNx + KW6002: 0.40 ± 0.03 ml/100gr rat, $p < 0.05$; Fig.2A) and MCS (Sham: 1.07 ± 0.02 vs. dAIH + vehicle: 0.74 ± 0.02, dAIH + KW6002: 0.71 ± 0.02, dNx + vehicle: 0.72 ± 0.03, dNx + KW6002: 0.72 ± 0.02 ml/100gr rat, $p < 0.05$; Fig.2B), but there were no significant differences among C₂HS groups ($p > 0.05$).

Three days post-dAIH, (i.e. 17 days post-C₂HS), dAIH + vehicle and dAIH + KW6002-treated rats had significantly increased V_T versus time control (TC) rats (dNx + vehicle and dNx + KW6002) when breathing room air (dAIH + vehicle: 0.47 ± 0.01, dAIH + KW6002: 0.46 ± 0.02 vs. dNx + vehicle: 0.40 ± 0.01, dNx + KW6002: 0.38 ± 0.03 ml/100 g, $p < 0.05$; Fig.2A). Although this effect is maintained 24 days post-injury, it was no longer significantly different from time controls. TC rats showed considerable spontaneous recovery, which was apparent at 24 days and continued up to the 38 days post-injury; at this time, V_T in control, untreated rats was no longer significantly different from sham rats without C₂HS (Fig. 2A, $p > 0.05$). During MCS, dAIH-treated rats showed significantly increased V_T at 17 and 24 days post-C₂HS versus TC rats (dAIH + vehicle: 0.83 ± 0.01 vs. dNx + vehicle: 0.73 ± 0.01, dNx + KW6002: 0.72 ± 0.01 ml/100 gr, all $p < 0.001$; Fig 2B), but this was not affected significantly by A_{2A} receptor inhibition (dAIH + vehicle: 0.83 ± 0.01 vs. dAIH + KW6002: 0.80 ± 0.02 ml/100gr, $p > 0.05$; Fig. 2B). At later time-points, there were no significant differences among groups, either when breathing room air or during MCS ($p > 0.05$).

Daily acute intermittent hypoxia does not affect respiratory frequency

Respiratory frequency (breaths per minute, bpm) significantly increased one day post-C₂HS versus pre-injury or sham values in all groups breathing air (dAIH + vehicle: 105 ± 2, dAIH + KW6002: 101 ± 2, dNx + vehicle: 102 ± 2, dNx + KW6002: 105 ± 3 bpm versus Sham: 90 ± 2 bpm, all $p < 0.05$; Fig.3A) and during MCS (dAIH + vehicle: 148 ± 2, dAIH + KW6002: 146 ± 2, dNx + vehicle: 148 ± 2, dNx + KW6002: 147 ± 3 bpm versus Sham: 138 ± 3 bpm, all $p < 0.05$; Fig.3B); there were no significant differences among treatment groups with C₂HS ($p > 0.05$).

Respiratory frequency remained greater than pre-injury values and sham rats at 17 days post-C₂HS during room air breathing (dAIH + vehicle: 109 ± 2, dAIH + KW6002: 107 ± 2, dNx + vehicle: 104 ± 2, dNx + KW6002: 105 ± 2 bpm vs. Sham: 92 ± 2 bpm, all $p < 0.05$; Fig.3A), and then progressively decreased so that, at 45 days post-C₂HS, no groups remained significantly different from sham values (dAIH + vehicle: 100 ± 2, dAIH + KW6002: 97 ± 2, dNx + vehicle: 96 ± 2, dNx + KW6002: 98 ± 2 vs. Sham: 93 ± 2 bpm, all $p > 0.05$; Fig.3A). Similarly, during MCS, respiratory frequency remained greater than pre-injury values and sham rats 17 days post-C₂HS (dAIH + vehicle: 149 ± 2, dAIH + KW6002: 151 ± 2, dNx + vehicle: 149 ± 2, dNx + KW6002: 150 ± 3 bpm vs. Sham: 140 ± 2 bpm, all $p < 0.05$; Fig.3B), and then remained greater than sham through the study.

Daily acute intermittent hypoxia does not induce significant ipsilateral motor recovery

Ipsilateral (injured) diaphragm and second external intercostal (T2 EIC) EMG activity were measured one-day post-C₂HS breathing air (Fig. 4,5). Inspiratory amplitude decreased

significantly versus pre-injury values and sham rats in diaphragm (Sham: $103 \pm 4.1\%$ of pre-injury values; dAIH + vehicle: $16.9 \pm 1.4\%$, dAIH + KW6002: $12.9 \pm 1.3\%$, dNx + vehicle: $13.5 \pm 2.8\%$, dNx + KW6002: $12.1 \pm 1.6\%$, all $p < 0.05$ when comparing sham vs C₂HS groups; Fig. 4A, 5D) and T2 EIC (Sham: $101.2 \pm 2.1\%$ of pre-injury values; dAIH + vehicle: $11.0 \pm 1.2\%$, dAIH + KW6002: $9.2 \pm 1.6\%$, dNx + vehicle: $8.2 \pm 2.0\%$, dNx + KW6002: $9.7 \pm 3.0\%$, all $p < 0.05$ comparing sham vs C₂HS groups; Fig. 4B, 5D); there were no significant differences among C₂HS groups.

Cervical hemisection was consistent among groups. Reconstruction of the hemisection (C₂HS) demonstrated a similar cross-sectional injury area in all groups (expressed as a percentage of total spinal cross sectional area; dAIH + vehicle: $48.2 \pm 1.3\%$, dAIH + KW6002: $46.3 \pm 1.0\%$, dNx + vehicle: $51.0 \pm 1.7\%$, dNx + KW6002: $45.4 \pm 1.5\%$, $p = 0.053$; Fig. 5E).

Ipsilateral diaphragm shows greatly reduced EMG amplitude ($\sim 13\%$ of pre-injury values) 17 days post-C₂HS, with a small ($\sim 20\%$ pre-injury values), but not significant spontaneous increase at the end of the study (52 days post-C₂HS, $p > 0.05$; Fig 4A). This result is consistent with previous studies showing modest ipsilateral phrenic activity after C₂HS (Dougherty et al., 2012b). In contrast, ipsilateral T2 EIC is less affected in any group (dAIH + vehicle: $78.5 \pm 3.1\%$, dAIH + KW6002: $77.1 \pm 2.1\%$, dNx + vehicle: $72.4 \pm 2.0\%$, dNx + KW6002: $73.5 \pm 2.9\%$ of pre-injury values, $p < 0.001$) 17 days post-C₂HS, with a non-significant trend to increase ($\sim 86\%$ of pre-injury values) by the end of experiments (52 days post-C₂HS, $p > 0.05$; Fig. 4B). Overall, dAIH alone or dAIH combined with A_{2A} receptor inhibition had no significant effect on ipsilateral diaphragm or T2 EIC EMG amplitude at any time-point (1-7 weeks, $p > 0.05$).

Daily acute intermittent hypoxia induce contralateral diaphragm and T2 intercostal recovery

Contralateral (uninjured) diaphragm and T2 EIC muscles had increased EMG amplitude (above pre-injury values) one-day post-C₂HS, demonstrating that mechanisms compensating for loss of ipsilateral function begin early after spinal injury. EMG amplitudes were significantly greater than pre-injury values and sham rats in right diaphragm (dAIH + vehicle: $125.6 \pm 2.8\%$, dAIH + KW6002: $128.0 \pm 3.8\%$, dNx + vehicle: $123.5 \pm 3.5\%$, dNx + KW6002: $128.2 \pm 2.5\%$ vs. Sham: $103.3 \pm 2.3\%$ of pre-injury values, $p < 0.05$; Fig 6A) and right T2 EIC (dAIH + vehicle: $112.3 \pm 1.5\%$, dAIH + KW6002: $113.7 \pm 2.7\%$, dNx + vehicle: $111.8 \pm 2.5\%$, dNx + KW6002: $113.4 \pm 2.2\%$ vs. Sham: $99.0 \pm 1.7\%$ of pre-injury values, $p < 0.05$; Fig 6B); there were no significant differences among C₂HS groups. Contralateral (uninjured) diaphragm maintained a 20-30% increase in EMG amplitude above pre-injury values in all groups at all times post-C₂HS (Fig. 6A, $p < 0.05$). In contrast, contralateral T2 EIC EMG amplitude returned to pre-injury values 17 days post-injury in TC rats (Fig. 6B, $p > 0.05$).

Daily AIH significantly increased EMG amplitude in contralateral diaphragm 17 days post-C₂HS versus control rats (dAIH + vehicle: $136.3 \pm 1.6\%$ vs dNx + vehicle: $124.5 \pm 1.2\%$ of pre-injury values, $p < 0.001$; Fig 6A). At the same time-point, dAIH also significantly increased T2 EIC EMG amplitude versus control rats (dAIH + vehicle: $110.5 \pm 1.7\%$ vs.

dNx + vehicle: $102.8 \pm 1.7\%$ of pre-injury values, $p < 0.001$; Fig. 6). At later time-points there were no significant differences between dAIH and control-treated rats in contralateral diaphragm or T2 EIC muscle.

Adenosine 2A receptor inhibition impairs dAIH-enhancement of contralateral diaphragm but not intercostal activity

In contralateral (uninjured) diaphragm, dAIH plus KW6002-treated rats showed significantly decreased EMG activity versus dAIH plus vehicle-treated rats at 17 days post-C₂HS (dAIH + vehicle: $136.3 \pm 1.6\%$ vs. dAIH + KW6002: $131.0 \pm 0.8\%$ of pre-injury values, $p = 0.015$; Fig. 6A). Thus, KW6002 impaired the functional benefits of dAIH at this early time post-injury, suggesting that respiratory motor recovery in diaphragm is A_{2A} receptor dependent. In contrast, in contralateral T2 EIC muscle, there were no statistically significant differences between dAIH plus vehicle and dAIH plus KW6002-treated rats at 17 days post-C₂HS (dAIH + vehicle: $110.5 \pm 1.7\%$, vs. dAIH + KW6002: $109.5 \pm 1.9\%$ of pre-injury values, $p = 0.601$; Fig. 6B).

Taken together, dAIH increases activity in both contralateral diaphragm and T2 EIC muscles. Although the dAIH effect was reduced by A_{2A} receptor inhibition in diaphragm, this treatment had no effect on dAIH-enhanced T2 EIC motor activity. Thus, dAIH-enhanced motor activity is A_{2A} modulated in diaphragm, but not in T2 EIC activity. Furthermore, weekly AIH (reminder) was unable to maintain dAIH-increased motor activity at later time-points in either right diaphragm or T2 EIC muscle.

Variable response to maximum chemoreceptor stimulation

Pre-injury EMG recordings and simultaneous plethysmography showed a robust MCS response (Fig. 5A) in both diaphragm and T2 EIC, as demonstrated previously (Navarrete-Opazo and Mitchell, 2014). During MCS one-day post-C₂HS, ipsilateral (injured) diaphragm and T2 EIC muscle activity were abolished (Fig. 5B). The MCS response was also abolished in contralateral (uninjured) T2 EIC (Fig. 5B), and was significantly reduced in contralateral diaphragm, compared to pre-injury values and sham rats at one-day post-injury (dAIH + vehicle: $91.63 \pm 1.1\%$, dAIH + KW6002: $94.68 \pm 1.93\%$, dNx + vehicle: $91.02 \pm 1.58\%$, dNx + KW6002: $93.18 \pm 2.34\%$ vs. Sham: $101.2 \pm 2.8\%$ of pre-injury values, all $p < 0.05$; Fig. 7A); there were no significant differences among C₂HS groups ($p > 0.05$).

Despite the extensive spontaneous recovery of ipsilateral T2 EIC muscle activity during room air breathing, the response with MCS was absent throughout the study; this effect may be explained by a “ceiling effect” where the ipsilateral muscle has fully utilized compensatory mechanisms and is unable to increase motor activity further. Similarly, the ipsilateral diaphragm response to MCS was abolished, except during the last measurement (52 days post-C₂HS), with a variable response ranging from -13.4% to 23.5% of pre-injury values (Fig. 7B, $p > 0.05$).

An MCS response in contralateral T2 EIC muscle is observed 38 days post-injury, with variable amplitude ranging from 13 to 71% of pre-injury values, and no significant differences among C₂HS groups. Contralateral diaphragm exhibited an attenuated MCS response at all time-points versus pre-injury values and sham rats (Fig. 7A, $p < 0.001$).

Daily AIH-treated rats significantly improved the MCS response in contralateral diaphragm 17 days post-injury versus time control rats (dAIH + vehicle: $97.1 \pm 1.2\%$ vs. dNx + vehicle: $90.1 \pm 1.1\%$, dNx + KW6002: $87.85 \pm 1.3\%$ of pre-injury values, $p < 0.001$; Fig.7A). This effect was significantly impaired by A_{2A} receptor inhibition (dAIH + vehicle: $97.1 \pm 1.2\%$ vs. dAIH + KW6002: $93.2 \pm 1.0\%$ of pre-injury values, $p = 0.015$; Fig. 7A), suggesting that dAIH-induced motor recovery in contralateral diaphragm is adenosine versus serotonin-dependent with acute C₂HS. At later time-points, contralateral MCS responses were not different among C₂HS groups.

Daily acute intermittent hypoxia does not affect forelimb grooming behavior

All rats exhibited normal grooming behavior (score=5, Fig.8) after telemetry implantation but before spinal injury. One day post-spinal injury, injured rats show complete paralysis of the left (injured) limbs; these rats were able to groom only with the right (uninjured) forelimb, but not the left (injured) forelimb (i.e. score = 0). This effect is consistent with complete cervical hemisection. All rats showed normal right forelimb grooming at all times post-C₂HS. At 17 days post-injury, half of the rats in all groups (dAIH + vehicle: n=4 rats, dAIH + KW6002: n=5, dNx + vehicle: n=2, dNx + KW6002: n=3) were able to touch the bottom of the snout with left forelimb (score of 1); this effect may reflect limited spontaneous improvement in trunk function (and balance) versus somatic recovery. The remaining rats in each group showed no improvement in grooming behavior with left forelimbs (i.e. score 0). Thus, dAIH had no demonstrable benefit in grooming behavior using the affected limb in rats with acute C₂HS.

Discussion

In this study, there were six major findings: 1) spontaneous motor recovery was modest in ipsilateral diaphragm, but substantial in ipsilateral T2 EIC when breathing room air, suggesting a greater contribution of inspiratory intercostals muscles (vs. diaphragm) to spontaneous recovery of respiratory function after SCI; 2) contralateral diaphragm and T2 EIC made more substantial contributions to spontaneous functional recovery, and these contributions were rapid (beginning one day post-C₂HS); 3) the contralateral diaphragm shows a consistent but reduced response to MCS, possibly due to a “ceiling effect” for contributions to spontaneous functional recovery from the uninjured diaphragm; 4) dAIH improves V_T when breathing room air and during MCS, but this effect could not be maintained by weekly AIH “reminders;” 5) dAIH primarily improves contralateral (uninjured), but not ipsilateral (injured) diaphragm and T2 EIC activity; and 6) A_{2A} receptor inhibition impairs dAIH-induced functional recovery of contralateral diaphragm, but not T2 EIC muscle activity. Thus, dAIH-induced functional recovery of diaphragm activity is adenosine- and not serotonin-dependent after acute C₂HS, and is accounted for by effects on respiratory motor output contralateral to injury.

Altered breathing pattern after C2 hemisection

After C₂HS, ipsilateral diaphragm paralysis reduces V_T and increases breathing frequency. This breathing pattern shift is consistent with previous reports in unanesthetized (Fuller et al., 2005; Fuller et al., 2006) and anesthetized rats (Golder et al., 2001b). Changes in

breathing pattern after C₂HS initially result from diminished pre-motor drive to respiratory motor neurons, thus reducing V_T . Frequency changes may reflect decreased feedback from lung stretch receptors (Golder et al., 2001a), and/or altered afferent inputs to the CNS that were disrupted by the injury (Golder et al., 2011). A prominent role for the latter mechanism is revealed in rats with cervical contusion injuries (which show similar pattern shifts). When these rats undergo vagotomy, they continue to exhibit elevated breathing frequencies (Golder et al., 2011). After cervical SCI, time-dependent increases in V_T occur, reflecting spontaneous spinal plasticity (Dougherty et al., 2012b; Lovett-Barr et al., 2012). Similarly, V_T was substantially reduced one-day post-C₂HS, yet exhibited remarkable spontaneous recovery by 24 days post-C₂HS (Fig.2A). Although breathing frequency was significantly higher than in sham rats, it slowly declined after 24 days post-C₂HS (Fig.3A). Thus, changes in breathing frequency are at least partially reversible, and vary inversely with V_T .

As reported previously (Fuller et al., 2005; Fuller et al., 2006; Fuller et al., 2009), rats with SCI exhibit deficits in ventilatory capacity when challenged with combined hypercapnia/hypoxia (i.e. MCS); thus, C₂HS causes respiratory deficits characterized by an inability to respond appropriately to respiratory challenges. This deficit is characterized by diminished ability to increase V_T during MCS (Fig. 2B), with minimal effect on breathing frequency (Fig. 3B).

Spontaneous recovery of ipsilateral motor activity

C₂HS disrupts bulbospinal inputs to spinal respiratory motor neurons distal to the lesion. However, partial return of ipsilateral diaphragm/intercostal activity occurs over a period of weeks to months after C₂HS (Dougherty et al., 2012a; Dougherty et al., 2012b; Fuller et al., 2006; Mantilla et al., 2013; Nantwi et al., 1999; Vinit et al., 2006). The extent that crossed phrenic phenomenon translates into a functional increases in respiratory capacity is questionable since at least three studies suggest that this spontaneous plasticity contributes minimally to tidal volume generating capacity up to 8 weeks post injury (Dougherty et al., 2012b; Fuller et al., 2006; Golder et al., 2003). After C₂HS, the ipsilateral (injured) phrenic nerve and/or hemidiaphragm show little inspiratory activity during normoxia (Dow et al., 2009; Golder and Mitchell, 2005) and we confirm this finding in diaphragm activity in unanesthetized rats. In contrast, the ipsilateral T2 EIC muscle showed remarkable recovery, reaching near normal levels in agreement with previous studies (Dougherty et al., 2012a; Sherrey and Megirian, 1990). Thus, accessory respiratory muscles may make a relatively greater contribution to preserving/restoring breathing capacity after cervical spinal injury. Further studies are warranted to determine mechanisms underlying enhancement of intercostal muscle EMG activity after C₂ spinal injury.

Removal of inhibitory reflexes may dis-inhibit intercostal motor neurons after spinal injury. In dogs, diaphragm paralysis rapidly increases activity of the inspiratory intercostal muscles in compensation (De Troyer, 1998). The increase may be due to diminished sensory afferent inputs arising coursing through the phrenic nerve that normally inhibit inspiratory intercostal activity (De Troyer, 1998). Indeed, bilateral stimulation of C₅ phrenic afferent neurons decreased intercostal inspiratory activity by 50% (De Troyer, 1998). In our C₂ hemisection

model, one side of the diaphragm is paralyzed, most likely decreasing afferent feedback and disinhibiting of inspiratory output in the intercostal muscles.

Plasticity in contralateral (uninjured) respiratory motor output has been observed previously after unilateral spinal injury (Katagiri et al., 1994; Mantilla et al., 2013; Rowley et al., 2005; Sherrey and Megirian, 1990; Teitelbaum et al., 1993). With C₂HS, right diaphragm and T2 EIC muscle activity increased, reflecting a form of compensatory respiratory plasticity. Presumably muscles on the uninjured side now make a greater proportional contribution to breathing, at least shortly after injury. Contralateral diaphragm EMG amplitude increased ~25% above pre-injury values, and remained elevated throughout the study (Fig. 6A). Similar compensatory (contralateral) plasticity was observed in right T2 EIC muscle activity (Fig. 6B), but this compensation had diminished 24 days post-injury, coinciding with recovery of the left (injured) T2 EIC. Although this reciprocal relationship suggests a causal relationship between these events, its mechanistic basis is not yet clear.

Daily acute intermittent hypoxia induces respiratory functional recovery

Daily AIH improves V_T 17 days post-injury when breathing room air, and up to 24 days post-injury during MCS (Fig.2B). This effect was not maintained by weekly AIH “reminders,” suggesting that more frequent repetitive AIH may be necessary to preserve long-lasting functional benefits. To some extent, the initial dAIH-induced recovery was masked by spontaneous recovery during normoxia, leading to a convergence between treated and untreated animals; thus, dAIH accelerated recovery, but did not lead to a sustained advantage. Since, respiratory frequency did not show significant differences across time, (room air breathing or MCS), spontaneous recovery of V_T and frequency arise from distinct mechanisms.

Increased ventilatory capacity induced by dAIH is likely attributable to increased contralateral motor activity in diaphragm and T2 EIC. A previous study reported that AIH augmented crossed spinal synaptic pathways (phrenic long-term facilitation; pLTF) at 8 weeks, but not 2 weeks, post-injury (Golder and Mitchell, 2005). The timing of recovery in the ability to elicit pLTF below the injury coincides with recovery of serotonin terminal density in the phrenic motor nucleus below the injury. Consistent with this study, we now show that dAIH did not increase motor activity in ipsilateral diaphragm or T2 EIC. In contrast, dAIH contributed to further compensatory plasticity on the uninjured side (both diaphragm and T2 EIC muscles), where serotonergic innervation of phrenic and thoracic motor nuclei remained intact.

A_{2A} receptor inhibition impairs dAIH-enhanced diaphragm activity

One week of dAIH, beginning 1-week post-C₂HS, is reported to restore ipsilateral phrenic motor output and breathing capacity in unanesthetized rats (Lovett-Barr et al., 2012), a time when serotonergic innervation of the phrenic motor nucleus remains disrupted (Golder and Mitchell, 2005). Because of this, the observed effects of dAIH may arise from mechanisms that are serotonin-independent. Indeed, KW6002, an A_{2A} receptor antagonist, impairs dAIH-induced recovery of diaphragm activity contralateral to injury at 17 days post-C₂HS. In contrast, the lack of any effect of KW6002 on dAIH-induced motor recovery in uninjured

T2 EIC suggests that adenosine is not initiating dAIH induced plasticity in this muscle/motor neuron group. Similarly, T2 EIC LTF in uninjured rats is unaffected by A_{2A} receptor inhibition (Navarrete-Opazo et al., 2014). Differential mechanisms (with respect to adenosine) giving rise to dAIH induced recovery of diaphragm versus intercostal motor activity remain to be explored. It is possible that spinal interneurons play a greater role in functional recovery of intercostal versus phrenic motor neurons, or that there is less disruption of serotonergic innervation in the thoracic spinal cord.

This is the first evidence that dAIH induces functional recovery of breathing capacity via adenosine-dependent mechanisms with acute SCI, since dAIH-induced recovery of diaphragm EMG activity was impaired by daily KW6002 pre-treatment. In recent unpublished studies, we also found that dAIH-induced recovery of breathing capacity after acute C₂HS does not require serotonin receptor activation (Terada, Vinit, MacFarlane and Mitchell, unpublished observations). Thus, dAIH induced functional recovery results from adenosine-dependent, serotonin-independent mechanisms with acute SCI (2 weeks), a time when serotonergic innervation below the site of injury is minimal (Golder and Mitchell, 2005).

Despite the effects of KW6002 on dAIH-induced functional recovery of diaphragm activity, we did not see similar impairment of dAIH effects on tidal volume. This difference may be explained by shifts to other inspiratory muscles that are less or unaffected by A_{2A} receptor inhibition. Since scalenus medius and intercostal muscles of the cephalic spaces T1, T2 T3 are inspiratory in rats (Megirian et al., 1987), dAIH may increase their motor output (as in T2 EIC); alternately, dAIH may induce respiratory activity in intercostal muscles that are not normally active. The differential effects of KW6002 on tidal volume versus diaphragm function after dAIH strongly suggests that other inspiratory muscles make greater contributors to ventilatory capacity after dAIH and SCI.

Variable maximum chemoreceptor response after acute spinal injury

Numerous studies confirm that ipsilateral phrenic nerve activity (below chronic C₂HS) increases during chemoreceptor challenge (Dougherty et al., 2012b; Fuller et al., 2006; Fuller et al., 2009). These observations are consistent with the hypothesis that the CPP primarily enables respiratory behaviors requiring large tidal volumes, such as a sigh or augmented breath (Golder et al., 2003). However, we found minimal (and variable) increase in ipsilateral diaphragm activity 52 days post-injury. Surprisingly, the MCS response was abolished in contralateral T2 EIC muscle for up to 38 days post-C₂HS, and was significantly reduced in contralateral diaphragm versus pre-injury values. We speculate that contralateral respiratory motoneurons are recruited after hemisection to compensate for loss of ipsilateral phrenic and thoracic activity and, therefore, are subject to a “ceiling effect” where they cannot increase motor output further. Injured T2 EIC motor recovery may have a functional significance during quiet breathing, but not during respiratory challenge after acute C₂HS. In contrast, uninjured diaphragm showed a reduced but consistent MCS response at all time-points, demonstrating that contralateral diaphragm has great functional significance during respiratory challenge. More importantly, dAIH improved breathing (i.e. tidal volume) during MCS 17 days post-injury.

Daily acute intermittent hypoxia does not improve automatic grooming behavior

The failure of dAIH to improve automatic grooming behavior differs in some respects from other studies demonstrating improved limb function in both rats and humans (Lovett-Barr et al., 2012; Trumbower et al., 2012). Even a single AIH presentation increases ankle strength (plantar flexion torque) in humans with motor incomplete chronic spinal injuries (Trumbower et al., 2012). Furthermore, dAIH (beginning 4 week post-C₂HS) improves forelimb function in injured rats (Lovett-Barr et al., 2012). In this latter study the authors mention that it was somewhat difficult to discriminate the effects of AIH *per se* versus paired training. Thus, our study may differ from that of Lovett-Barr and colleagues (2012) in that the dAIH was applied shortly after C₂HS (beginning 1 versus 4 weeks post-injury), the latter study used combined treatment (dAIH plus daily ladder walking) and the specific tasks differ in their demands on the animal (automatic grooming versus ladder walking). Considering that dAIH mainly increases contralateral versus ipsilateral diaphragm and T2 EIC function, the lack of ipsilateral forelimb recovery in acute C₂HS rats is not unexpected. However, we cannot rule out that the grooming test may not be sensitive enough to detect subtle somatic improvements elicited by dAIH.

Conclusion

Collectively, we found that dAIH improves respiratory function in unanesthetized rats with acute cervical spinal injuries; however, the differential effects (dAIH versus normoxia treated rats) are transient, reflecting slow spontaneous recovery without treatment. Weekly AIH reminders are insufficient to preserve relative functional recovery (versus time controls), suggesting that more robust repetitive AIH protocols are necessary to preserve functional advantages of dAIH. Thus, the main benefit of dAIH was to accelerate functional recovery of breathing capacity when applied shortly after spinal injury.

At this time post-injury, the main functional benefits of dAIH are attributable to increased EMG activity in contralateral (uninjured) diaphragm and ipsilateral T2 EIC muscle versus ipsilateral diaphragm—a more frequently studied outcome. Further, A_{2A} receptor inhibition actually impairs the extent of functional recovery induced by dAIH of contralateral diaphragm, suggesting that adenosinergic mechanisms play a key role in the therapeutic effects of repetitive AIH with acute SCI. Systemic administration of selective A_{2A} receptor agonists (CGS21680) minimize tissue damage, locomotor dysfunction and inflammatory profiles when administered within 24 hours post-SCI (Genovese et al., 2009; Paterniti et al., 2011). Although systemic A_{2A} receptor inhibition undermined functional benefits of dAIH with acute injury, we hypothesize that it will enhance the benefits of dAIH with chronic SCI, after spinal serotonergic innervation has recovered. This possibility remains to be explored.

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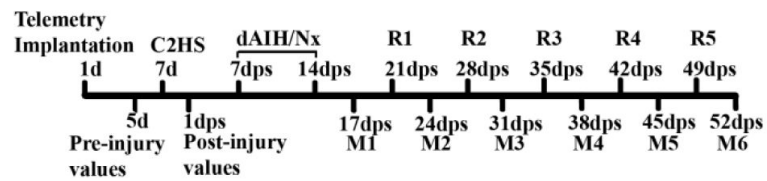


Fig. 1.

Timeline describing the experimental design from day 1 (1d) until 52 days post-spinal cord injury (dps). C2HS: cervical hemisection in second segment, dAIH/dNx: daily acute intermittent hypoxia or normoxia for seven days depending on groups (see methods), R1-R5: weekly presentations or “reminders” of acute intermittent hypoxia or normoxia, M1-M6: weekly measurements including grooming test and simultaneous plethysmography and electromyography of bilateral diaphragm and second external intercostal muscles.

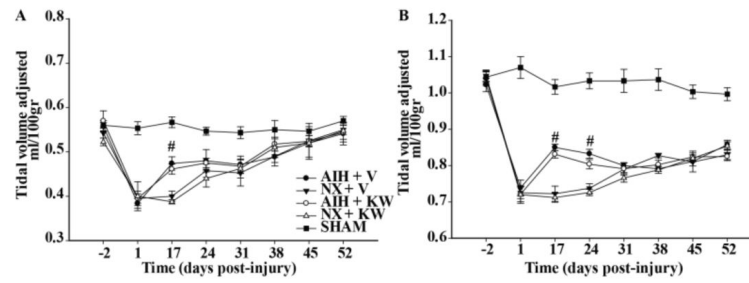


Fig. 2.

Absolute values of tidal volume (V_T) per 100 gr rat during normoxia (Nx, A) and maximum chemoreceptor stimulation (MCS, B) in all groups 2 days before spinal injury and then, 1 day up to 52 days post-injury. Note: (1) all groups show reduced V_T 1 dps in A and B compared to shams; (2) AIH elicits an increase in V_T during Nx and MCS, which was not significantly affected by adenosine A_{2A} antagonist. AIH: acute intermittent hypoxia, V: vehicle (DMSO), KW: KW6002, Nx: normoxia. Values are means \pm SEM. # significantly different from controls (Nx + V and Nx + KW6002); $p < 0.05$.

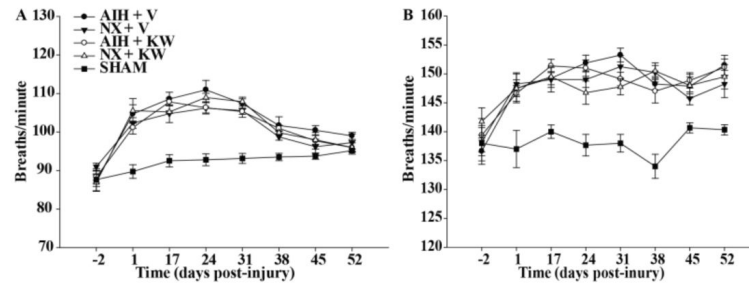


Fig. 3.

Absolute values of respiratory frequency during normoxia (Nx, A) and maximum chemoreceptor stimulation (MCS, B) in all groups studied 2 days before spinal cord injury, and one day up to 52 days post-injury. Respiratory frequency is significantly increased in all groups, compared to sham during normoxia and MCS. AIH: acute intermittent hypoxia, V: vehicle (DMSO), KW: KW6002.

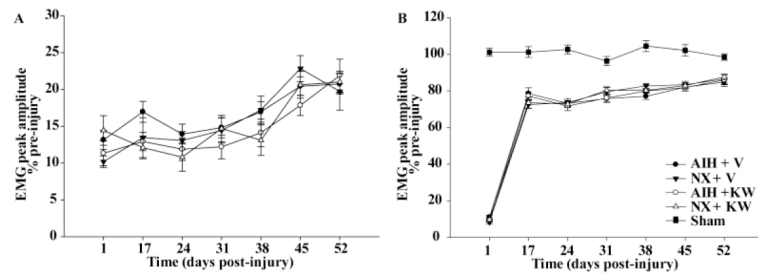


Fig. 4.

Changes in ipsilateral (injured) diaphragm (A) and second external intercostal muscle (T2 EIC, B) muscle amplitude during normoxia (Nx) expressed as percent change of pre-injury values one day up to 52 days post-injury. The spontaneous recovery is remarkable in ipsilateral T2 EIC in all groups (B) and not significant in ipsilateral diaphragm (A). Daily acute intermittent hypoxia (dAIH) alone or combined with A_{2A} inhibition (KW6002) does not have an effect in neither diaphragm nor T2 EIC ipsilateral motor recovery. AIH: acute intermittent hypoxia, V: vehicle (DMSO), KW: KW6002.

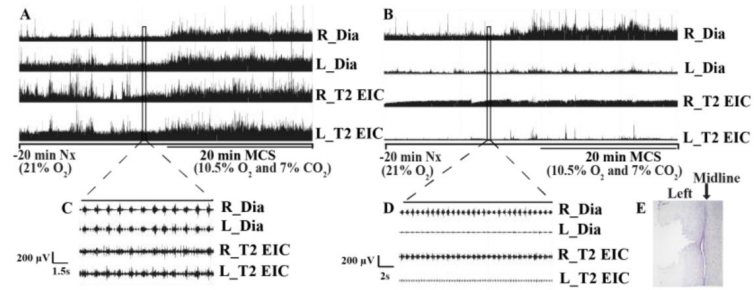


Fig. 5.

Representative integrated (A and B) and raw (C and D) EMG activity of right/left diaphragm (R/L_Dia) and second external intercostal muscle (R/L_T2 EIC) before (A, C) and one day after (B, D) spinal hemisection (C2HS) during maximum chemoreceptor stimulation (MCS). Note: (1) significantly reduced EMG activity in ipsilateral diaphragm and T2 external intercostal in (D), confirming the cervical hemisection; (2) absence of MCS response in injured (left) diaphragm and bilateral T2 EIC muscle in (B). (E) Representative longitudinal spinal cord slide (C1-C6) stained with Cresyl violet showing C2HS.

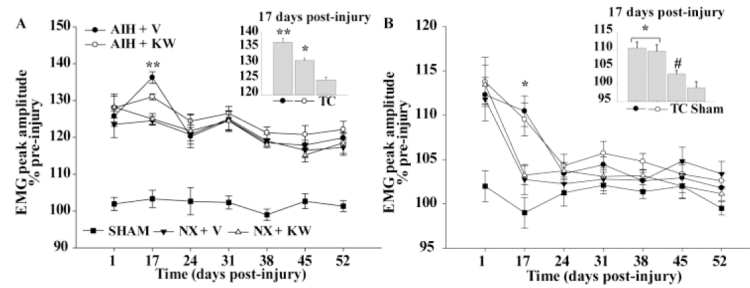


Fig. 6.

Changes in contralateral (uninjured) diaphragm (A) and second external intercostal muscle (T2 EIC, B) muscle amplitude during normoxia (Nx) expressed as percent change of pre-injury values one day up to 52 days post-injury. Note: (1) all groups show significant increase in EMG amplitude compared to sham and pre-injury values during Nx in the diaphragm (A), demonstrating compensatory plasticity; (2) contralateral T2 EIC exhibits a transitory compensatory plasticity in all groups at 17 days post-injury; (3) daily acute intermittent hypoxia (dAIH) significantly increases EMG amplitude at 17 days post-injury in diaphragm and T2 EIC and this effect is impaired by A_{2A} inhibition in diaphragm but not in T2 EIC muscle. AIH: acute intermittent hypoxia, V: vehicle (DMSO), KW: KW6002. Values are mean \pm SEM. ** significantly different from AIH + KW6002, * significantly different from time controls (TC: Nx + V and Nx + KW6002), # significantly different from sham; $p < 0, 05$.

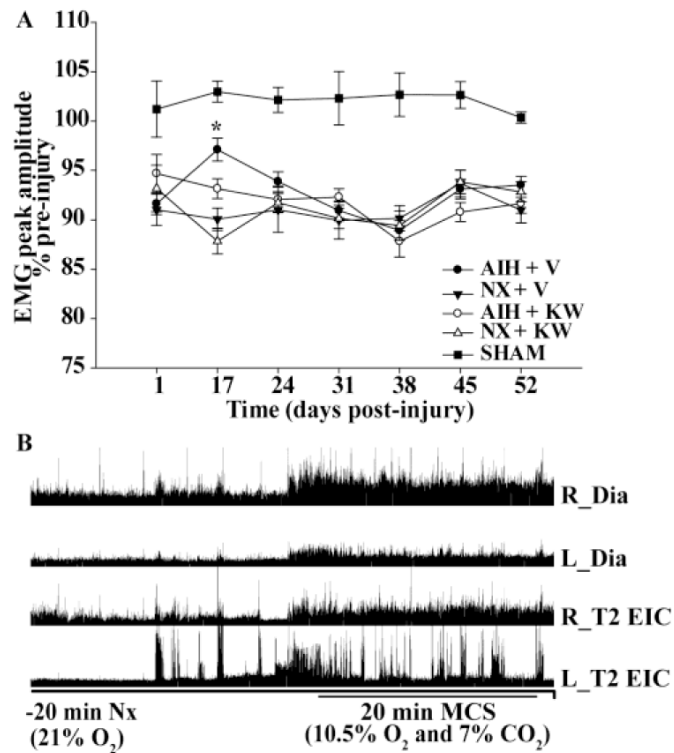


Fig. 7. Changes in contralateral (uninjured) diaphragm during maximum chemoreceptor stimulation (MCS, A) and representative integrated EMG activity of right/left diaphragm (R/L_Dia) and second external intercostal muscle (R/L_T2 EIC) at 52 days post-injury during MCS (B). Note: (1) reduced but consistent MCS response in all groups in uninjured diaphragm (A); (2) daily acute intermittent hypoxia (dAIH) significantly increases EMG amplitude at 17 days post-injury and this effect is impaired by KW6002; (3) small MCS response in uninjured T2 EIC (B); (4) the MCS response in injured T2 EIC remains abolished at all this time-points (B).

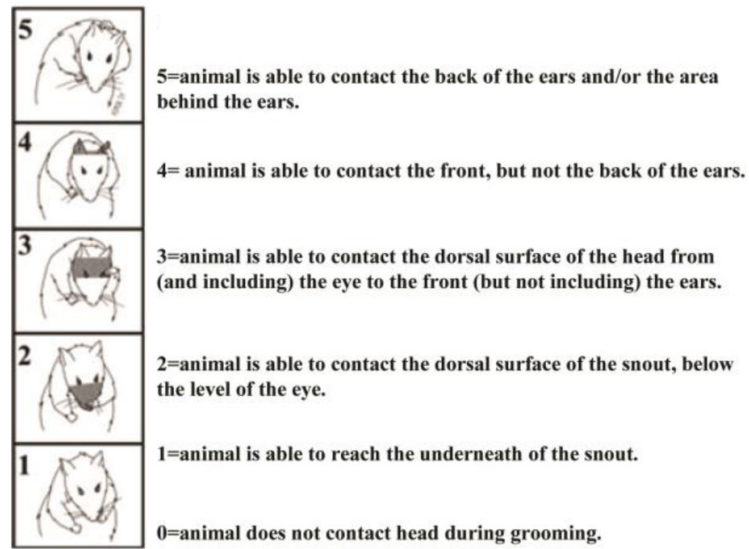


Fig 8.
Grooming Test scoring.